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(54) Title: GENE ENCODING THE HUMAN HOMOLOG OF MAD2 (57) Abstract <p>This invention provides isolated nucleic acid encoding human MAD2, isolated human MAD2 protein. This invention further provides a method of detecting the presence of MAD2 in a tissue sample, a method of determining whether a tumor is susceptible to treatment with a mitotic spindle inhibitor by detecting the presence of MAD2 in the tumor and a method of suppressing tumor formation in a subject which comprises administering the nucleic acid encoding human MAD2 to the subject in an amount effective to enhance expression of MAD2. This invention also provides a nucleic acid reagent capable of detecting the MAD2 gene or gene product and a method for <i>in situ</i> identification of tumors which may be susceptible to treatment with mitotic spindle inhibitors by detecting the absence of nucleic acid encoding MAD2 in the tumor.</p>		

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GENE ENCODING THE HUMAN HOMOLOG OF MAD2

5 This application claims the benefit of copending U.S. Provisional Application Serial No. 60/001,736, filed August 1, 1995.

10 The invention disclosed herein was made in the course of work under NCI Core Grant No. 08748 from the National Cancer Institute and NSF Grant No. IBN-9118977 from the National Science Foundation. Accordingly, the U.S. Government has certain rights in this invention.

15 Throughout this application, various publications are referenced by Arabic numerals in brackets. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are in their entirety hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background of the Invention

25 MAD2 is a mitotic checkpoint gene whose function is required for yeast cells to arrest before undergoing cell division if the mitotic spindle apparatus is improperly attached to the chromosomes. (Li and Murray). In the absence of functional MAD2 protein, yeast cells which are exposed to drugs which inhibit the formation of a mitotic spindle, such as benomyl, vinblastine, nocodazole, etc. 30 undergo rapid cell death due to massive chromosome loss. Yeast cells which have a functional MAD2 protein can survive such drug treatment because they are able to stop dividing prior to the chromosome loss event.

35

The interest in the MAD2 gene stems from the possibility that tumor cells that are hypersensitive to

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chemotherapeutic agents which inhibit the formation of the mitotic spindle may be sensitive to these drugs precisely because they are defective in the MAD2 checkpoint. Analysis of the MAD2 status of a given tumor may therefore be a predictor of chemosensitivity. In addition, the loss of MAD2 function in a normal cell may predispose that cell to aberrant chromosome segregation events, a hallmark of tumor progression.

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Summary of the Invention

This invention provides isolated nucleic acid encoding human MAD2 protein.

5

This invention also provides a vector comprising the nucleic acid encoding human MAD2 protein and a host cell containing the vector.

10 This invention also provides a nucleic acid probe comprising a nucleic acid molecule comprising at least 15 nucleotides capable of specifically hybridizing with a unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid encoding human MAD2
15 protein.

This invention further provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding human MAD2 protein so as to
20 prevent translation of the mRNA. This invention further provides a vector comprising the antisense oligonucleotide and a host cell containing the vector.

25 This invention also provides isolated human MAD2 protein.

This invention also provides an antibody capable of specifically binding to the isolated human MAD2 protein.

30 This invention further provides a method of detecting the presence of human MAD2 protein in a sample which comprises:

- a) contacting the sample with the antibody capable of specifically binding to the isolated human MAD2 protein, under conditions permitting the
35 formation of a complex between the antibody and the human MAD2 protein in the sample; and
- b) detecting the complex formed in step (a),

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thereby detecting the presence of human MAD2 protein in the sample.

5 This invention further provides a method of detecting the expression of MAD2 in a sample which comprises:

- 10 a) contacting the sample with the nucleic acid probe comprising a nucleic acid molecule comprising at least 15 nucleotides capable of specifically hybridizing with a unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid encoding human MAD2 protein, under conditions permitting the hybridization of the probe to any of the RNA present in the sample; and
- 15 b) detecting the presence of the hybridized probe, a positive detection indicating the expression of MAD2 in the sample.

20 This invention further provides a method of determining the susceptibility of a tumor sample to treatment with a mitotic spindle inhibitor by detecting the presence of human MAD2 protein in the tumor which comprises steps of:

- 25 a) contacting the tumor sample with the antibody capable of specifically binding to human MAD2 protein, under conditions permitting formation of a complex between the antibody and the human MAD2 protein in the tumor sample; and
- 30 b) detecting the complex formed in step (a), the presence of the complex indicating that the tumor is susceptible to treatment with a mitotic spindle inhibitor.

35 This invention also provides a method of determining whether a tumor is susceptible to treatment with a mitotic spindle inhibitor by detecting the presence of MAD2 protein in the tumor which comprises:

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- 5 a) contacting a tumor sample with the nucleic acid probe comprising a nucleic acid molecule comprising at least 15 nucleotides capable of specifically hybridizing with a unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid encoding human MAD2 protein, under conditions permitting the hybridization of the probe to the RNA present in the sample; and
- 10 b) detecting the presence of the hybridized probe, a positive detection indicating susceptibility to treatment with a mitotic spindle inhibitor.

15 This invention also provides a pharmaceutical composition comprising an amount of the nucleic acid encoding human MAD2 protein capable of passing through a cell membrane effective to enhance the expression of MAD2 and a suitable pharmaceutically acceptable carrier.

20 This invention further provides a method of suppressing tumor formation in a subject which comprises administering the nucleic acid encoding human MAD2 protein to the subject in an amount effective to enhance expression of MAD2. This invention also provides a

25 method of suppressing tumor formation in a subject which comprises administering the pharmaceutical composition to the subject.

30 This invention also provides a pharmaceutical composition comprising an amount of the antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding for human MAD2 protein so as to prevent translation of the mRNA, which is capable of passing through a cell membrane and effective to inhibit the

35 expression of MAD2 and a suitable pharmaceutically acceptable carrier.

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This invention also provides a nucleic acid reagent capable of detecting the MAD2 gene or gene product.

5 This invention also provides a method for in situ identification of tumors which may be susceptible to treatment with mitotic spindle inhibitors by detecting the absence of nucleic acid encoding MAD2 in the tumor which method comprises contacting the tumor with a
10 suitably labeled nucleic acid reagent capable of detecting the MAD2 gene or gene product.

Other uses and objectives of this invention will apparent to those of ordinary skill in the art in view of the Detailed Description which follows. Such other uses and
15 objectives are deemed to be within the scope of the claims which follow.

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Brief Description of the Figures

- 5
Figure 1: Side by side comparison of the yeast MAD2 and human MAD2 amino acid sequences.
- 10
Figures 2A and 2B: Side by side comparison of the yeast MAD2 and human MAD2 nucleic acid sequences.
- 15
Figure 3A-C: Characterization of human MAD2.
- 20
A Nucleotide and the predicted amino acid sequences of hSMAD2 cDNA. The amino acid sequence predicted by the hSMAD2 open reading frame is indicated in single-letter code. The stop codon is indicated with the asterisk.
- 25
B Alignment of the predicted hSMad2 protein sequence with those of *X. laevis* Mad2 and *S. cerevisiae* Mad2p. Amino acids identical in at least two of the three MAD2 proteins are boxed. Dashes indicate gaps.
- 30
C Human MAD2 encodes a 24 kD protein. Total protein extracts from HeLa cells (lanes 1, 3, and 5) or HeLa cells transiently transfected with pCMV5-hSMAD2 for 48 hr (lanes 2, 4, and 6) were resolved by 12% SDS-PAGE (30 μ g of protein per lane), transferred to nitrocellulose, and probed with the preimmune IgG (lanes 1 and 2), the α -hSMad2 Δ IgG (lanes 3 and 4) or the affinity-purified α -hSMad2 antibody (lanes 5 and 6). The positions of prestained kleidoscope molecular mass markers (in kilodaltons, Bio-Rad) are shown on the right.
- 35
Figure 4A1-4: Human MAD2 functions as a mitotic checkpoint gene.

HeLa cells electroporated with α -hSMad2 antibodies fail

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to arrest in mitosis in the presence of nocodazole. HeLa cells were electroporated with buffer, the preimmune IgG the α -hsMad2 Δ IgG, or the affinity-purified α -hsMad2 antibody as indicated. Electroporated cells were allowed to attach to the plates for 6 hours and then treated with 200 nM nocodazole for additional 18 hours before being photographed.

Figure 5A(1-6)-B(1-12): Subcellular localization of hsMad2 in HeLa cells.

A1-6 Subcellular localization of hsMad2 during interphase. HeLa cells were stained with the preimmune IgG, the α -hsMad2 Δ IgG, or the affinity-purified α -hsMad2 antibody as indicated (top row). DNA was visualized with DAPI (bottom row). Cells were observed with a 40x oil immersion objective.

B1-12 Subcellular localization of hsMad2 during mitosis. HeLa cells were triple stained with affinity-purified α -hsMad2 antibody (top row), human α -centromere serum (middle row), and DAPI (bottom row). Cells in prometaphase (PM), arrested in prometaphase by nocodazole treatment (NOC), metaphase (M), and anaphase (A) are shown. Cells were observed with a 100x oil immersion objective.

Figure 6A-B: T47D cells fail to arrest in mitosis in response to nocodazole treatment and are defective for hSMAD2 expression.

A T47D and RH1 fail to undergo mitotic arrest upon nocodazole treatment. Exponentially growing HeLa, F65, T47D, and RH1 cells were treated with 100 nM nocodazole and harvested at the indicated time points. Cells were transferred to slides by cytopinning, stained with DAPI, and then scored for their mitotic indices (M.I.). For each cell line, three independent experiments were performed and the average M.I. is shown.

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B T47D is defective for hsMAD2 expression. Equal amounts of total protein extracts from the indicated cell lines were resolved by 12% SDS-PAGE, transferred to
5 nitrocellulose, and probed with α -hsMad2 serum (1:500 dilution).

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Detailed Description of the Invention

This invention provides isolated nucleic acid encoding human MAD2 protein. The nucleic acid of this invention
5 can be DNA or RNA. In separately preferred embodiments when the nucleic acid is DNA it may be genomic DNA or cDNA. In another preferred embodiment when the nucleic acid is DNA it has a nucleic acid sequence substantially similar to the nucleic acid sequence of Figure 3A. In a
10 further embodiment, the DNA sequence is as set forth in Figure 3A. In another preferred embodiment when the nucleic acid is RNA it may be mRNA.

The DNA molecules of the subject invention also include
15 DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues
20 specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties
25 of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate
30 DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful
35 for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors,

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transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

5

In one embodiment of the invention the cDNA is labeled with a detectable moiety. Substances which function as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a
10 fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase,
15 horseradish peroxidase, β -galactosidase, fluorescein or streptavidin/biotin.

In a preferred embodiment the nucleic acid of the subject invention encodes a protein having an amino acid sequence
20 substantially similar to the amino acid sequence of Figure 3A.

This invention also provides a replicable vector comprising the nucleic acid encoding human MAD2 protein
25 and a host cell containing the vector. In one embodiment the host cell is a prokaryotic or eukaryotic cell. In an embodiment wherein the the host cell is a prokaryotic, it is a bacterial cell. In still another embodiment wherein the host cell is a eukaryotic cell, the host cell may be
30 a yeast, insect, plant, or a mammalian cell.

Numerous vectors for expressing the inventive proteins may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other
35 viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus,

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polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also be needed for optimal synthesis of mRNA. These additional elements may include splice signals, as well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

These vectors may be introduced into a suitable host cell to form a host vector system for producing the inventive proteins. Methods of making host vector systems are well known to those skilled in the art.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast

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cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as
5 hosts, including, but not limited to, the mouse fibroblast cell NIH-3T3 cells, CHO cells, HeLa cells, Ltk⁻ cells and COS cells. Mammalian cells may be transfected by methods well known in the art such as calcium phosphate precipitation, electroporation and
10 microinjection.

This invention further provides a method of producing a polypeptide having the biological activity of the MAD2 protein which comprising growing host cells of a vector
15 system containing the MAD2 protein sequence under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.

This invention also provides a nucleic acid probe comprising a nucleic acid molecule comprising at least 15 nucleotides capable of specifically hybridizing with a
20 unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid encoding human MAD2 protein.

25 In a preferred embodiment the nucleic acid probe comprises DNA. In an additionally preferred embodiment the nucleic acid probe comprises RNA.

30 This invention further provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding human MAD2 protein so as to prevent translation of the mRNA. This invention further provides a replicable vector comprising the antisense
35 oligonucleotide and a host cell containing the vector.

In one embodiment the antisense oligonucleotide has a

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sequence capable of specifically hybridizing to mRNA so as to prevent translation of the mRNA. In the practice of this invention the antisense oligonucleotide may be contained within a replicable vector. In the practice of
5 this invention the vector may be contained within a host cell. In one embodiment the host cell is a prokaryotic or eukaryotic cell. In an embodiment wherein the host cell is a prokaryotic, it is a bacterial cell. In still another embodiment wherein the host cell is a eukaryotic
10 cell, the host cell may be a yeast, insect, plant, or a mammalian cell.

This invention also provides isolated human MAD2 protein. In the preferred embodiment the isolated human MAD2
15 protein has an amino acid sequence substantially similar to the amino acid sequence shown in Figure 3A.

This invention also provides an antibody capable of specifically binding to the isolated human MAD2 protein.
20 In a preferred embodiment the antibody is capable of specifically binding to the protein having the amino acid sequence shown in Figure 3A.

In one embodiment the antibody is a monoclonal antibody.
25 In a separate embodiment the antibody is a polyclonal antibody.

Polyclonal antibodies may be produced by injecting a host animal such as rabbit, rat, goat, mouse or other animal
30 with the immunogen of this invention. The sera are extracted from the host animal and are screened to obtain polyclonal antibodies which are specific to the immunogen. Methods of screening for polyclonal antibodies are well known to those of ordinary skill in
35 the art such as those disclosed in Harlow & Lane, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: 1988) the contents

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of which are hereby incorporated by reference.

5 The monoclonal antibodies may be produced by immunizing
for example, mice with an immunogen. The mice are
inoculated intraperitoneally with an immunogenic amount
of the above-described immunogen and then boosted with
similar amounts of the immunogen. Spleens are collected
from the immunized mice a few days after the final boost
and a cell suspension is prepared from the spleens for
10 use in the fusion.

Hybridomas may be prepared from the splenocytes and a
murine tumor partner using the general somatic cell
hybridization technique of Kohler, B. and Milstein, C.,
15 Nature (1975) 256: 495-497. Available murine myeloma
lines, such as those from the American Type Culture
Collection (ATCC) 12301 Parklawn Drive, Rockville, MD
20852 USA, may be used in the hybridization. Basically,
the technique involves fusing the tumor cells and
20 splenocytes using a fusogen such as polyethylene glycol.
After the fusion the cells are separated from the fusion
medium and grown in a selective growth medium, such as
HAT medium, to eliminate unhybridized parent cells. The
hybridomas may be expanded, if desired, and supernatants
25 may be assayed by conventional immunoassay procedures,
for example radioimmunoassay, using the immunizing agent
as antigen. Positive clones may be characterized further
to determine whether they meet the criteria of the
invention antibodies.

30

Hybridomas that produce such antibodies may be grown in
vitro or in vivo using known procedures. The monoclonal
antibodies may be isolated from the culture media or body
fluids, as the case may be, by conventional
35 immunoglobulin purification procedures such as ammonium
sulfate precipitation, gel electrophoresis, dialysis,
chromatography, and ultrafiltration, if desired.

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In the practice of the subject invention the antibodies can be labeled with a detectable moiety. As noted above, a "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or streptavidin/biotin.

This invention further provides a method of detecting the presence of human MAD2 protein in a sample which comprises:

- a) contacting the sample with the antibody, wherein the antibody is labeled with a detectable moiety and is capable of specifically binding to a human MAD2 protein, under conditions permitting the formation of a complex between the antibody and the human MAD2 protein in the sample; and
- b) detecting the complex formed in step (a), thereby detecting the presence of human MAD2 protein in the sample.

In a preferred embodiment the detection in step (b) is performed by detection of a detectable moiety on the antibody which may be a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or streptavidin/biotin.

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As used herein, "sample" means body tissue or fluid, including but not limited to blood, urine, saliva, and cerebrospinal fluid.

5 This invention further provides a method of detecting the expression of MAD2 in a sample which comprises:

- 10 a) contacting the sample with a nucleic acid probe, wherein the probe is labeled with a detectable moiety and comprises at least 15 nucleotides capable of specifically hybridizing with a unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid encoding human MAD2 protein, under conditions permitting the hybridization of the probe to the RNA present in the sample; and
- 15 b) detecting the presence of the hybridized probe, a positive detection indicating the expression of MAD2 in a sample.

20

The term "probe" as used herein refers to any nucleic acid molecule which can be labeled and which forms a double helix by binding with a molecule containing a nucleic acid sequence of interest through complementary base paring. Those skilled in the art also refer to such probes as "hybridization probes." For example, when using a DNA probe to locate a DNA sequence of interest, a sample containing double stranded DNA can be reacted with the DNA probe to locate any DNA molecule in a sample which comprises the sequence of interest ("target DNA"). In such methods, the double stranded DNA in the sample is disassociated into its single strands and then reacted with a DNA probe. The probe binds to any target DNA in the sample by complementary base paring, i.e., adenine matches with thymidine and guanine with cytosine. The DNA probe, therefore, is a single strand of a DNA double helix which comprises nucleic acid molecules which are

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complementary to the sequence of interest.

5 Methods of making labeled nucleic acid probes, both DNA and RNA, are well known to those of ordinary skill in the art.

10 As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence sufficiently similar to its own so as to form double-helical segments through hydrogen bonding between complementary base pairs. As used herein, a "unique sequence" is a sequence specific to only the nucleic acid molecules encoding the human MAD2 protein.

15 In a preferred embodiment the detection in step (b) is performed by detection of a detectable moiety on the probe which may be a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or
20
25 steptavidin/biotin.

This invention further provides a method of determining the susceptibility of a tumor sample to treatment with a mitotic spindle inhibitor which comprises steps of:

- 30 a) contacting the tumor sample with an antibody, wherein the antibody is labeled with a detectable moiety and is capable of specifically binding to a human MAD2 protein, under conditions permitting the formation of a complex between the antibody and the human
35 MAD2 protein in the tumor sample; and
b) detecting the complex formed in step (a), the

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presence of the complex indicating that the tumor is susceptible to treatment with a mitotic spindle inhibitor.

5 In a preferred embodiment the detection in step (b) is performed by detection of a detectable moiety on the antibody which may be a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a
10 secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or streptavidin/biotin.

15 This invention also provides a method of determining whether a tumor is susceptible to treatment with a mitotic spindle inhibitor by detecting the presence of MAD2 protein in the tumor which comprises:

- 20 a) contacting a tumor sample with a nucleic acid probe, wherein the probe is labeled with a detectable moiety and comprises at least 15 nucleotides capable of specifically hybridizing with a unique sequence included
25 within the sequence of the isolated nucleic acid encoding for human MAD2, under conditions permitting the hybridization of the probe to the RNA present in the sample; and
- 30 b) detecting the presence of the hybridized probe, a positive detection indicating susceptibility to treatment with a mitotic spindle inhibitor.

35 In a preferred embodiment the detection in step (b) is performed by detection of a detectable moiety on the probe which may be a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent

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label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or steptavidin/biotin.

This invention also provides a pharmaceutical composition comprising an amount of the nucleic acid encoding human MAD2 protein capable of passing through a cell membrane effective to enhance the expression of MAD2 and a suitable pharmaceutically acceptable carrier.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stensic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

In addition to the standard characteristics of the pharmaceutically acceptable carriers, the "suitable" carriers of the subject are further characterized as being able to penetrate the cell membrane. Therefore in one embodiment of the pharmaceutical composition the pharmaceutically acceptable carrier binds to a receptor on a cell capable of being taken up by the cell after binding to the structure.

In a preferred embodiment of the pharmaceutical

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composition the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a selected tumor cell type.

- 5 This invention further provides a method of suppressing tumor formation in a subject which comprises administering the nucleic acid encoding human MAD2 protein to the subject in an amount effective to enhance expression of MAD2. This invention also provides a
10 method of suppressing tumor formation in a subject which comprises administering the pharmaceutical composition to the subject.

15 In the practice of this invention, the administration of the nucleic acid or pharmaceutical composition comprising the nucleic acid may be effected by any of the well known methods including, but not limited to, oral, intravenous, intraperitoneal, intramuscular or subcutaneous or topical administration. Topical administration can be effected
20 by any method commonly known to those skilled in the art and include, but are not limited to, incorporation of the pharmaceutical composition into creams, ointments or transdermal patches.

- 25 This invention also provides a pharmaceutical composition comprising an amount of the antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding for human MAD2 protein so as to prevent translation of the mRNA, which is capable of passing
30 through a cell membrane and effective to inhibit the expression of MAD2 and a suitable pharmaceutically acceptable carrier.

35 In a preferred embodiment the pharmaceutical composition comprises an amount of the antisense oligonucleotide capable of passing through a cell membrane and effective to inhibit the expression of MAD2 and a suitable

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pharmaceutically acceptable carrier.

5 In a particularly preferred embodiment the pharmaceutical composition the oligonucleotide is coupled to a substance which inactivates mRNA. Examples of such "substances" include, but are not limited to, ribozymes. In this embodiment the pharmaceutically acceptable carrier may be capable of binding to a receptor on a cell capable of being taken up by the cell after binding to the structure. In this embodiment of the pharmaceutical composition the pharmaceutically acceptable carrier may additionally be capable of binding to a receptor which is specific for a selected tumor cell type.

15 Finally, this invention also provides a nucleic acid reagent capable of detecting the MAD2 gene or gene product. The nucleic acid reagent can be used in a method for in situ identification of tumors which may be susceptible to treatment with mitotic spindle inhibitors by detecting the absence of nucleic acid encoding MAD2 in the tumor. Such method comprises contacting the tumor with a suitably labeled nucleic acid reagent capable of detecting the MAD2 gene or gene product.

25 In the practice of this aspect of the invention the suitably labeled nucleic acid reagent comprises a detectable moiety chosen from the group consisting of a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label and a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, β -galactosidase, fluorescein or streptavidin/biotin.

35 This invention provides a recombinant non-human vertebrate animal wherein functional hSMAD2 protein is

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not expressed. As used herein, recombinant animals are the animals or their ancestors, which have been manipulated by recombinant technology. In an embodiment, the animal is a rodent. In a preferred embodiment, the rodent is a mouse. These recombinant animals will be useful for study of tumorigenesis. Methods to make these animals are known in the art. Sometimes these animals may be called "knock-out" animal as the gene coding for hSMAD2 is rendered nonfunctional.

10

The following Experimental Details are provided to aid in the understanding of the invention. The Experimental Details are not intended, and should not be interpreted, to limit the scope of the invention which is more fully defined in the claims which follow.

15

Experimental Details

First Series of Experiments

- 5 Example 1: Isolation of human homologue of MAD2 as a high-copy number suppressor of *cbf1Δ*.

10 Budding yeast strain YNN415 requires exogenous methionine for growth and is supersensitive to the microtubule-destabilizing drug thiabendazole due to the *cbf1* null allele. As a means of identifying human clones that could substitute for *cbf1*, YNN415 was first transformed with a human cDNA library which carries the LEU2 marker by LiCl method (Guthrie and Fink, 1991). About one half
15 million transformants were planted on ten SD-met-leu plates. After 5-day incubation at 30°C, 19 colonies grew up and were subsequently tested for thiabendazole sensitivity on YPD plates containing 100 ug/ml thiabendazole. Among these 19 clones, only one clone
20 grew well on both SD-met-leu and YPD+thiabendazole plates. Plasmid DNA from this clone was then recovered by standard methods (Maniatis et al., 1982) and its cDNA insert was sequenced by the dideoxynucleotide method (Maniatis et al., 1982). Analysis of the DNA sequence of
25 the 1.5 kb cDNA insert showed that it contained an open reading frame that encodes a protein of 205 amino acids. We used this 205 amino acid sequence to search the Genbank database and found that only the budding yeast MAD2 showed significant homology to our cDNA clone. The
30 overall protein sequence identity between our clone and yeast MAD2 is about 40% and the overall similarity is about 60%. Therefore, based on the sequence homology, we named our gene human MAD2 (*hsmad2*). Retransformation of *hsmad2* into YNN415 showed a weak but reproducible
35 complementation activity of the thiabendazole sensitivity. Although not wishing to be limited to any particular theory, it is believed that *hsmad2* will

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function similar to yeast MAD2 which has been shown to function as a spindle assembly checkpoint in cell cycle M phase. For example, hSMAD2 may monitor the kinetochore-spindle attachment before anaphase occurs.

5

Example 2: Generation of anti-MAD2 antibody and chromosomal mapping of hSMAD2.

10 Full-length hSMAD2 coding sequence was subcloned into the pET28(a) so that a histidine tag was fused in-frame to the N terminus of hSMAD2 protein. Full-length his-hSMAD2 was then overexpressed and purified following manufacturer's instructions. Purified his-hSMAD2 protein was then injected into New Zealand White rabbit to
15 generate anti-hSMAD2 serum. We showed the specificity of our anti-hSMAD2 serum by immunoprecipitating the in vitro translated MAD2 protein and demonstrating that the antibody binding is completed efficiently by the purified protein. Briefly, 1 microgram of MAD2 mRNA was incubated
20 with rabbit reticulocyte lysates (Promega) in the presence of 1 mM amino acids minus methionine and 10 microcuries 35-S-methionine for 1.5 hours at 30°C in a 50 microliter volume. Five microliters of the reaction was then diluted into 150 microliters of RL-150 buffer
25 (Benezra et al., 1990) and various dilutions of the antisera added (with and without competing polypeptides) before precipitating the complexes with protein A agarose beads (Repligen). The immunoprecipitates were then analyzed by standard SDS-PAGE (Maniatis et al., 1982).
30 By Western blotting (performed as described in Harlow and Lane, 1988) we have shown that anti-hSMAD2 serum specifically recognizes a polypeptide that migrates on SDS-polyacrylamide gels with the expected molecular weight. Transient transfection (performed by the DEAE-dextran method as described in Maniatis et al., 1982) and
35 peptide competition assays showed that the above-mentioned polypeptide corresponds to the endogenous

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hsMAD2 protein. Immunostaining of Hela cells fixed in 4% paraformaldehyde and permeabilized in 0.35% Triton-X-100 was performed by standard methods (Harlow and Lane, 1988) and showed that our anti-hsMAD2 serum can specifically stain certain regions in nuclei that may correspond to the centromeres of chromosomes. This very unusual staining pattern can now be used as a marker for proper MAD2 deposition. Alterations in this pattern, in addition to changes in MAD2 protein levels by Western analysis, can be used to monitor aberrant MAD2 function.

Example 3: Tumor suppression.

To determine whether hsMAD2 functions as a tumor suppressor gene we determined the chromosomal locus of hsMAD2. A P1 human genomic clone that contains hsMAD2 was isolated and used to hybridize to human chromosomes by FISH (fluorescent in situ hybridization). Preliminary data indicates that hsMAD2 maps to a region thought to contain a tumor suppressor locus. One breast tumor line examined (T47D) showed very high sensitivity to taxol and had decreased MAD2 mRNA and protein levels.

Experimental Discussion

The human MAD2 gene was found by a genetic selection procedure carried out in yeast designed to identify molecules which could suppress the sensitivity of a particular strain of yeast to mitotic spindle inhibitors. By overexpressing random protein coding sequences from a human glioma cDNA library in this yeast strain, we were able to select for a yeast cDNA and protein. The h-MAD2 gene can partially suppress the benomyl sensitivity of a mutant yeast strain lacking functional yeast MAD2 directly demonstrating that the human clone is functionally related to the yeast clone. Despite the

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similarity of the yeast and human protein sequences (see Figure 1) the nucleotide sequences are sufficiently diverged (see Figure 2) that use of the yeast sequence in analysis would be impossible.

References of the First Series of Experiments

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- 10 3. Maniatis, T. et al. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Press, Cold Spring New York).
4. Li, R. and Murray, A.W. (August 1, 1991). Cell 66: pp. 519-531.

Second Series of Experiments

A cDNA clone which encodes the human homologue of the product of *Saccharomyces cerevisiae* mitotic checkpoint gene *MAD2* was isolated. In yeast, this gene product is required for cells to arrest in mitosis if the mitotic spindle assembly is perturbed (1). HeLa cells electroporated with an affinity purified antibody against human *MAD2* protein fail to undergo mitotic arrest in the presence of the microtubule depolymerizing drug nocodazole demonstrating directly that h*MAD2* is a necessary component of the mitotic checkpoint in human cells. Immunofluorescence analysis of HeLa cells indicates that during mitosis, the h*MAD2* protein is localized at the kinetochore after chromosome condensation but is no longer observed at the kinetochore when the chromosomes are aligned at the metaphase plate suggesting that h*MAD2* might be a sensor of mitotic spindle attachment. Finally, T47D, a breast tumor cell line which is hypersensitive to taxol and nocodazole treatment, is unable to execute the mitotic checkpoint and has reduced h*MAD2* expression. This result suggests that defects in h*MAD2* may play a role in the observed sensitivity of certain tumors to mitotic spindle inhibitors.

During mitosis, the onset of anaphase is demarcated by the separation of sister chromatids and the destruction of cyclin B which are irreversible events that commit a cell to complete the division cycle (2-4). Mitotic checkpoint control mechanisms (5-8) have evolved which test the cell's preparedness to undergo division and block cell cycle progression prior to the irreversible events associated with anaphase when the mitotic spindle apparatus is not appropriately assembled. For example, defects in the structure of the mitotic spindle, unoccupied microtubule binding sites at the kinetochores,

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and a lack of tension on the kinetochores supplied by opposing forces on bioriented metaphase chromosomes can all activate the mitotic checkpoint and arrest cells in mitosis (1, 9-12). Progress has been made in identifying some of the molecular components that sense failures in mitotic spindle assembly and send the "stop mitosis" signal. In budding yeast, six non-essential genes have been identified that are required for the execution of the mitotic checkpoint: *MAD1-3* (1) and *BUB1-3* (9). These genes were identified in screens for mutant cells that are hypersensitive to mitotic spindle inhibitors. Studies in higher eukaryotes have clearly indicated the existence of a similar mitotic checkpoint pathway (10-14), but its molecular components have not yet been identified.

A human cDNA clone was isolated (15) in a screen for high copy number suppressors of the thiabendazole (a mitotic spindle inhibitor) sensitivity observed in yeast cells lacking *Cbflp*, a component of the budding yeast kinetochore (18-20). Sequence determination of the cDNA revealed an open reading frame of 205 amino acids (Fig. 3A) that was highly homologous to the product of the budding yeast mitotic checkpoint gene *MAD2* (Genbank accession number U14132). With the introduction of two very small gaps (1 and 2 residues), the two proteins are 40% identical and 60% similar over the entire open reading frame (Fig. 3B). The human locus is therefore referred to as *hsMAD2* (for *homo sapiens MAD2*). A *MAD2* homologue has also been identified and characterized in *Xenopus laevis* and the protein sequence alignment is shown in Figure 3B. The protein encoded by the *hsMAD2* cDNA has a predicted molecular weight of 23.5 kD with two potential amphipathic alpha helices at residues 64-74 and 124-134. The presence and relative positions of the amphipathic alpha helices are conserved between all three species. The fact that both *hsMAD2* and yeast *MAD2* can

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partially suppress the thiabendazole sensitivity of *cbf1* null yeast cells (data not shown) suggest the possibility that in the absence of *CBF1* the mitotic checkpoint is not fully activated.

5

To further characterize hsmad2, polyclonal antibodies were generated and affinity-purified (21). In addition, the IgG fractions from the preimmune serum and the α -hsmad2 serum that was first passed over the hsmad2 affinity column (referred to as α -hsmad2 Δ IgG) were also isolated (21). By Western analysis (23) the affinity-purified α -hsmad2 antibody specifically recognizes a single protein species of approximately 24 kD in total HeLa cell extracts that is not observed with either preimmune IgG or α -hsmad2 Δ IgG (Fig. 3C). Extracts from HeLa cells that have been transiently transfected with a hsmad2 expression vehicle show an increase in intensity of the 24 kD band indicating that this species is almost certainly encoded by the hsmad2 cDNA (Fig. 3C, compare lane 6 with lane 5). It is also clear from this analysis that the affinity-purified antibody against hsmad2 is highly specific for the protein expressed in human cells.

In order to determine if hsmad2 functions as a mitotic checkpoint gene, affinity-purified antibodies against hsmad2 were electroporated into HeLa cells and the status of the mitotic checkpoint was determined (24). If hsmad2 activity is required for the execution of the mitotic checkpoint, then the α -hsmad2 antibody would be expected to inhibit this activity and prevent mitotic arrest in the presence of mitotic spindle inhibiting drugs. As shown in Figure 4A, a significant percentage of cells electroporated with either buffer alone, the preimmune IgG or the α -hsmad2 Δ IgG are rounded up after the nocodazole treatment indicative of cells arrested in mitosis (also see below). In contrast, cells

electroporated with the α -hsMad2 antibody show far fewer rounded cells after nocodazole treatment (note that 80%~90% of the cells that survive the electroporation have taken up IgG as assayed by immunofluorescence, data not shown). In order to confirm that the α -hsMad2 antibody was quantitatively affecting the mitotic index (M.I.) of the cells exposed to nocodazole, the percentage of IgG⁺ cells in mitosis was determined after the nocodazole treatment of the electroporated cells. As shown in Table 1, whereas the average M.I. of the IgG⁺ cells electrophorated with either the preimmune IgG or the α -hsMad2 Δ IgG was about 30% (471/1588), the M.I. of IgG⁺ cells electroporated with the α -hsMad2 antibody was 1.8% (18/1016). This latter result is unlikely to be due to an arrest prior to the onset of mitosis since the cells electroporated with the α -hsMad2 antibody continue to cycle for 30 hours at the same rate as the cells electroporated with the preimmune IgG (data not shown). These data therefore directly demonstrate that hsMad2 is required in human cells for the execution of the mitotic checkpoint in response to nocodazole treatment. Since XMad2 has also been shown to be an essential component of the mitotic checkpoint in Xenopus laevis, it is concluded that the mitotic checkpoint function of MAD2 is highly conserved during evolution and probably plays a critical role in ensuring accurate chromosome segregation.

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Table 1. Summary of the antibody electroporation experiments.

	Experi- ment	Nocodazole (nM)	Time (hr)	Antibody	IgG+	IgG ⁺ mitotic cells
5						
10	1	100	12	Preimmune α -hsMad2	254 206	52 3
	2	200	18	Preimmune α -hsMad2	270 295	77 6
15	3	200	18	Preimmune α -hsMad2 Δ α -hsMad2	261 233 217	83 74 4
20	4	200	18	Preimmune α -hsMad2 Δ α -hsMad2	275 295 298	94 91 5

25 Four independent experiments have been performed. For preimmune IgG and α -hsMad2 Δ IgG electroporations, a total of 1588 IgG⁺ cells were counted among which 471 are mitotic cells, thereby giving an overall mitotic index of 30%. For α -hsMad2 IgG electroporation, 18 cells were found to be in M phase among 1016 IgG⁺ cells counted, giving a mitotic index of 1.8%.

30

Studies in budding yeast (25,26), insect and vertebrate cells (10-14) have pointed to a close link between the kinetochore and the mitotic checkpoint pathway. It was therefore of interest to determine the subcellular localization of hSMad2 (27). During interphase, hSMad2 distributes throughout cells with a non-uniform distribution pattern (Fig. 5A). Specifically, perinuclear and patchy cytoplasmic staining are consistently observed. In mitotic cells, the pattern of hSMad2 staining appears to vary with the stage of mitosis. hSMad2 colocalizes with the kinetochore in those cells in which the chromosomes are highly condensed but not yet aligned at the metaphase plate, presumably in either late prophase or prometaphase (Fig. 5B, this stage is referred to as prometaphase). At metaphase and anaphase however, hSMad2 staining is absent from the chromosomes (Fig. 5B). The kinetochore localization of hSMad2 in prometaphase (when few kinetochores are attached to the mitotic spindle) suggests that hSMad2 may function as a sensor of the spindle-kinetochore interaction and can activate the mitotic checkpoint when the interaction is incomplete. Consistent with this possibility, persistent kinetochore localization of hSMad2 in HeLa cells arrested in mitosis by nocodazole treatment which inhibits the spindle-kinetochore interaction has been observed (28) (Fig. 5B). It is possible that hSMad2 may also monitor other events such as the alignment of the chromosomes at the metaphase plate.

Since yeast cells defective in mitotic checkpoint genes are hypersensitive to mitotic spindle inhibitors, it was necessary to determine if the hypersensitivity to such drugs observed in certain human tumor cells could be accounted for by defects in mitotic checkpoint execution. T47D, a breast tumor cell line, and RH1, a rhabdomyosarcoma cell line, were found to be hypersensitive to taxol and nocodazole (data not shown).

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This sensitivity could be accounted for by their failure to undergo mitotic arrest in response to nocodazole treatment as shown in Fig. 6A. Additionally, T47D cells have been shown to be karyotypically unstable (29), consistent with the idea that they are defective in the mitotic checkpoint. Whether these two cell lines have any defects in hSMAD2 protein expression was examined. By Western analysis T47D has about a 3.5 fold reduction in hSMAD2 protein level relative to nocodazole and taxol resistant cell lines (Fig. 6B). RH1 cells on the other hand show no such decrease. This data suggests the possibility that an hSMAD2 defect in T47D contributes to the observed failure to undergo mitotic arrest in response to nocodazole treatment and the resultant hypersensitivity to this compound. In RH1 cells, the mitotic checkpoint defect is either in some other component of the pathway or due to a more subtle change in hSMAD2 activity.

It has been shown that the MAD proteins in budding yeast are required for accurate chromosome segregation under normal growth conditions (1). It is reasonable therefore that loss of hSMAD2 function might lead to aberrant chromosome segregation in mammalian cells, an event which leads to genomic instability and has been shown to be associated with tumor formation in a number of cell types (30). This hypothesis can now be tested by the generation and analysis of MAD2 null mice.

30

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15. YNN415 (18) (the *cbf1* null strain) was transformed with a human cDNA library (kindly provided by Dr. John Colicelli) using a lithium acetate method (16).
5 Approximately 4×10^5 transformants were plated on YPD plates containing 100 $\mu\text{g/ml}$ thiabendazole (Sigma). After a 6-day incubation at 30°C, 19 clones were isolated and retested for thiabendazole resistance. The thiabendazole resistance of one clone was
10 dependent on the plasmid bearing the human cDNA. Plasmid DNA isolated from this clone contains a 1.5 kb cDNA insert. Nucleotide sequence determination was performed by the dideoxy chain termination method (17) with Sequenase (US Biochemicals).
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25
20. Baker, R. & Masison, D. *Mol. Cell. Biol.* 10, 2458-2467 (1990).
21. Full-length hsMAD2 open reading frame was subcloned
30 into pET-28a(+). 6xHis-tagged hsMad2 was overexpressed in BL21 and purified on a Ni-NTA column according to the manufacturer's instructions (Ni-NTA; Qiagen). Polyclonal antibodies were prepared by injection of the purified fusion protein
35 into two female New Zealand White rabbits (HRP Inc., Pennsylvania). Purified 6xHis-tagged hsMad2 was coupled to CNBr-activated Sepharose 4B (Pharmacia)

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- 5 according to the manufacturer's instructions to generate the hsMad2 affinity column. The α -hsMad2 polyclonal serum was loaded onto the affinity column and the α -hsMad2 antibody was eluted from the column with 0.1 M glycine [pH 2.5] (22). A negative control antibody (α -hsMad2D IgG) was prepared by passing the flowthrough of the affinity column over a Protein A-Sepharose column (Pharmacia) and eluting the IgG fraction with 0.1 M glycine [pH 2.5] (22).
- 10 IgG from the corresponding preimmune serum was also isolated using a Protein A-Sepharose column (Pharmacia). All of the purified IgGs were extensively dialyzed against PBS and concentrated to 2 mg/ml (α -hsMad2 IgG and α -hsMad2 Δ IgG) or 1.5
- 15 mg/ml (preimmune IgG) using the Centricon-30 units (Amicon) according to the manufacturer's instructions.
- 20 22. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988).
- 25 23. Protein extracts were prepared by lysing cells in NP-40 lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, 50 mM NaF, 0.25 mM Na_3VO_4 , 1 mM PMSF, and 5 $\mu\text{g/ml}$ of aprotinin, antipain, pepstatin, and leupeptin). Protein extracts were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) as
- 30 described (22). Immunoblotting was performed using the enhanced chemiluminescence protocol (DuPont NEN) according to the manufacturer's instructions. The affinity purified antibodies were used at 0.8 $\mu\text{g/ml}$ and the donkey anti-rabbit IgG HRP (horseradish peroxidase)-linked secondary antibody (Amersham) was
- 35 used at 1:10,000.

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24. HeLa cells were split into fresh medium one day before electroporation. Cells were harvested, washed, and resuspended in PBS to 1×10^7 cells/ml. 1×10^6 cells were mixed with approximately 25 μ g of affinity-purified antibodies and incubated for 10 min at room temperature in 0.4 cm Gene Pulser cuvettes. The electric pulse was delivered from a Gene Pulser (Bio-Rad) set at 300 V, infinite resistance, 250 μ F. Immediately after the pulse, cells were transferred into 6-well dishes containing pre-warmed medium and allowed to firmly attach to the dishes for 6 hr. Cells were then exposed to either 100 nM or 200 nM nocodazole for 12 or 18 hr before being photographed. Cells were trypsinized off the dishes and transferred to slides by cytopinning at 500 rpm for 6 min. Cells were fixed and stained with DAPI and anti-rabbit IgG secondary antibody (27). IgG⁺ cells and IgG mitotic cells were counted by immunofluorescence microscopy (27).
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27. Cells were fixed at -20°C with 100% methanol for 6 min and permeabilized at -20°C with 100% acetone for 30 s. Cells were then washed with PBS and blocked with 3% BSA in PBS for 1 hr. For staining of hSMad2, affinity purified antibody was used at 2 μ g/ml in the blocking buffer for 1 hr at room temperature. Cells were then washed six times with PBS containing 0.1% Triton X-100 and incubated for 30 min with 1:50 diluted donkey anti-rabbit IgG FITC-conjugated secondary antibody (Amersham). After six washes in PBS, cells were stained with

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- 5 DAPI (0.1 μ g/ml in PBS), washed again, and mounted. For co-immunostaining of hsMad2 and centromeres, cells were incubated with both affinity-purified α -hsMad2 antibody (2 μ g/ml) and human α -centromere serum (1:100 diluted) derived from a scleroderma patient (a gift from Dr. Keith Elkon at Cornell University Medical Center) for 1 hr, washed as described above, and then incubated with donkey anti-rabbit IgG FITC-conjugated secondary antibody 10 (1:50, Jackson ImmunoResearch) and donkey anti-human IgG Rhodamine-labeled secondary antibody (1:50, Jackson ImmunoResearch). All cells were analyzed with a Zeiss Axiophot microscope.
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What is claimed is:

1. Isolated nucleic acid encoding human MAD2 protein.
- 5 2. The isolated nucleic acid of claim 1, wherein the nucleic acid is DNA.
3. The isolated nucleic acid of claim 2, wherein the DNA is genomic DNA.
- 10 4. The isolated nucleic acid of claim 2, wherein the DNA is cDNA.
- 15 5. The isolated nucleic acid of claim 4, wherein the nucleic acid has a nucleic acid sequence substantially similar to the nucleic acid sequence of Figure 3A.
- 20 6. The isolated nucleic acid of claim 1, wherein the nucleic acid is RNA.
7. The isolated nucleic acid of claim 6, wherein the nucleic acid is mRNA.
- 25 8. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a protein having an amino acid sequence substantially similar to the amino acid sequence of Figure 3A.
- 30 9. A vector comprising the nucleic acid of claim 1.
10. A host cell containing the vector of claim 9.
11. The host cell of claim 10, wherein the cell is a
35 prokaryotic or eukaryotic cell.
12. The host cell of claim 10, wherein the prokaryotic

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cell is a bacterial cell.

13. The host cell of claim 10, wherein the eukaryotic cell is a yeast, insect, plant, or mammalian cell.
- 5
14. A nucleic acid probe comprising a nucleic acid molecule comprising at least 15 nucleotides capable of specifically hybridizing with a unique nucleotide sequence included within the nucleotide sequence of the isolated nucleic acid of claim 1.
- 10
15. The nucleic acid probe of claim 14, wherein the nucleic acid probe is DNA.
- 15
16. The nucleic acid probe of claim 14, wherein the nucleic acid probe is RNA.
17. An antisense oligonucleotide having a sequence capable of specifically hybridizing to mRNA encoding human MAD2 protein so as to prevent translation of the mRNA.
- 20
18. A replicable vector comprising the antisense oligonucleotide of claim 17.
- 25
19. A host cell containing the vector of claim 18.
20. The host cell of claim 19, wherein the cell is a prokaryotic or eukaryotic cell.
- 30
21. The host cell of claim 19, wherein the prokaryotic cell is a bacterial cell.
22. The host cell of claim 19, wherein the eukaryotic cell is a yeast, insect, plant, or mammalian cell.
- 35
23. An isolated human MAD2 protein.

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24. The protein of claim 23, having an amino acid sequence substantially similar to the amino acid sequence of Figure 3A.
- 5 25. An antibody capable of specifically binding to human MAD2 protein.
26. The antibody of claim 25, wherein the antibody is a monoclonal antibody.
- 10 27. The antibody of claim 26, wherein the antibody is labeled with a detectable moiety.
- 15 28. The antibody of claim 26, wherein the detectable moiety is a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step.
- 20 29. A method of detecting the presence of human MAD2 protein in a sample which comprises:
- 25 a) contacting the sample with the antibody of claim 25, under conditions permitting the formation of a complex between the antibody and the human MAD2 protein in the sample; and
- b) detecting the complex formed in step (a), thereby detecting the presence of human MAD2 protein in the sample.
- 30 30. The method of claim 29, wherein the antibody is labeled with a detectable moiety.
- 35 31. The method of claim 30, wherein the detectable moiety is a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step.

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32. A method of detecting the expression of MAD2 in a sample which comprises:
- 5 a) contacting the sample with the nucleic acid probe of claim 14, under conditions permitting the hybridization of the probe to the RNA present in the sample; and
- b) detecting the presence of the hybridized probe, a positive detection indicating the expression of MAD2 in a sample.
- 10
33. The method of claim 32, wherein the probe is labelled with a detectable moiety.
34. The method of claim 33, wherein the detectable moiety is a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step.
- 15
35. A method of determining the susceptibility of a tumor sample to treatment with a mitotic spindle inhibitor which comprises steps of:
- 20 a) contacting the tumor sample with the antibody of claim 25, under conditions permitting formation of a complex between the antibody and the human MAD2 protein in the tumor sample; and
- 25 b) detecting the complex formed in step (a), the presence of the complex indicating that the tumor is susceptible to treatment with a mitotic spindle inhibitor.
- 30
36. The method of claim 35, wherein the antibody is labelled with a detectable moiety.
- 35
37. The method of claim 36, wherein the detectable moiety is a fluorescent label, a radioactive atom,

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a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step.

- 5 38. A method of determining whether a tumor is susceptible to treatment with a mitotic spindle inhibitor by detecting the presence of MAD2 protein in the tumor which comprises:
- 10 a) contacting the tumor sample with the nucleic acid probe of claim 14, under conditions permitting the hybridization of the probe to the RNA present in the sample; and
- 15 b) detecting the presence of the hybridized probe, a positive detection indicating susceptibility to treatment with a mitotic spindle inhibitor.
- 20 39. The method of claim 38, wherein the detectable moiety is a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step.
- 25 40. A pharmaceutical composition comprising an amount of the nucleic acid of claim 1 capable of passing through a cell membrane effective to enhance the expression of MAD2 and a suitable pharmaceutically acceptable carrier.
- 30 41. The pharmaceutical composition of claim 40, wherein the pharmaceutically acceptable carrier binds to a receptor on a cell capable of being taken up by the cell after binding to the structure.
- 35 42. The pharmaceutical composition of claim 40, wherein the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a

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selected tumor cell type.

- 5 43. A method of suppressing tumor formation in a subject which comprises administering the nucleic acid of claim 1 to the subject in an amount effective to enhance expression of MAD2.
- 10 44. A method of suppressing tumor formation in a subject which comprises administering the pharmaceutical composition of claim 40 to the subject.
- 15 45. A pharmaceutical composition comprising an amount of the antisense oligonucleotide of claim 17 which is capable of passing through a cell membrane and effective to inhibit the expression of MAD2 and a suitable pharmaceutically acceptable carrier.
- 20 46. The pharmaceutical composition of claim 45, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.
47. The pharmaceutical composition of claim 46, wherein the substance which inactivates mRNA is a ribozyme.
- 25 48. The pharmaceutical composition of claim 45, wherein the pharmaceutically acceptable carrier binds to a receptor on a cell capable of being taken up by the cell after binding to the structure.
- 30 49. The pharmaceutical composition of claim 45, wherein the pharmaceutically acceptable carrier is capable of binding to a receptor which is specific for a selected tumor cell type.
- 35 50. A nucleic acid reagent capable of detecting the MAD2 gene or gene product.

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51. A method for in situ identification of tumors which may be susceptible to treatment with mitotic spindle inhibitors by detecting the absence of nucleic acid encoding MAD2 in the tumor which comprises
5 contacting the tumor with a suitably labeled nucleic acid reagent of claim 50.
52. The method of claim 51, wherein the suitably labeled nucleic acid reagent comprises a detectable moiety
10 chosen from the group consisting of a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label and a label which may be detected through a secondary enzymatic or binding step.
- 15 53. A recombinant non-human vertebrate animal wherein functional hSMAD2 protein is not expressed.
54. The recombinant non-human vertebrate animal of claim
20 53, wherein the animal is a rodent.
55. The recombinant non-human vertebrate animal of claim 54, wherein the rodent is a mouse.

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FIG. 1

hu-Mad2
sc-Mad2

Malqlsr EOGITLERGS AEI VAEFF SFG INSIL YORG I YPSETE TRVQKYG
M----- SOSISLKGS TRT VTTEFF EYS INSIL YORG VYPAEDE VT VKKYD

hu-Mad2
sc-Mad2

LHLL VTTBLEL IKY LNNVVEQLKD MLYKCSVQ KLV VVISNIES GEV LERN
LHLL KTHDDEL KD YIRK ILLQVHR MLLGGKCN QLV LCI VDKDE GEV VERN

hu-Mad2
sc-Mad2

QEDIE -----C DKTAKD DSAPR eksqkal CDE IRSV IROIT AT VTFL PL
SENVQ hisgnsn GQDDV VDLNNT ----- CSQIR AL IROIT SS VTFL PE

hu-Mad2
sc-Mad2

L--EVSCSEDL IYTD KDLV VPEK WEE SGPF ITNSE EVRLRS TT TI HK
Ltk EGGYTE TVLA YTD AD AK VPLE WADSN SKETPDGEV VQFKT ESTNDHK

hu-Mad2
sc-Mad2

VNSMVA YKipvnd
VGAQVSYKY ----

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FIG. 2A

h-MAD2	atg g c g c t g c a g c t c t c c g g g a g c a g g a a t c a c c c t g c g g g a g c g c
y-MAD2	atg t c a c a a t c a a t a t c a c t a a a g g t t c a a c a g g a c a g t t a c a - - - -
h-MAD2	c g a a a t c g t g g c c g a g t t c t t c t c a t t c g g c a t c a a c a g c a t t t a t a t c
y-MAD2	- - - - - g a a t t t t t c g a g t a c a g c a t t a a t t c c a t t t g t a c c
h-MAD2	a g c g t g g c a t a t a t c c a t c t g a a a c c t t a c t c g a g t g c a g a a t a c g g a
y-MAD2	a a a g a g g c g t a t a c c c a g c a g a a g a t t t c g t a a c g g t g a a a a g t a c g a t
h-MAD2	c t c a c c t t g c t t g t a a c t a c t g a t c t t g a g c t a a a a t a c c t a a a t a a
y-MAD2	c t t a c g t t a c t a a a g a c a c a t g a t g a t g - - - - -
h-MAD2	t g t g g t g g a a c a a c t g a a a g a t t g g t a t a c a a g t g t c a g t t c a g a a c
y-MAD2	- - - - - a a c t g a a a g a t t a c a t c g g a a a a t t c t t c t a c a g t t c
h-MAD2	t g g t t g t a g t t a t c t c a a a t a t t g a a g t - - - - -
y-MAD2	a c a g g t g g c t t c t t g g t g g a a a a t g c a a c a a t t a g t a t t a t g t a t t g t a
h-MAD2	- - - - - g g t g a g g t c c t g g a a a g a t g g c a g t t t g a t a t t g a g t g
y-MAD2	g a c a a g g a t g a g g a g a g g t g g t g g a a a g a t g g t c c t t c a a t g t g c a a c a
h-MAD2	t g a c a a g a c t g c a a a g a t g a c a g t g c a c c c c a g a a a a g t c t c a g a a a g
y-MAD2	c a t t t c t g g c a a t a g c a c g g g c a g g a t g a t g t t g t a g a t t t a a a t a c a a

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FIG. 2B

h-MAD2	ctatccaggatg	AAATC	C	GTT	CAG	TGAT	CAG	ACA	GAT	CAC	AG	CT	ACG	GT	G	
y-MAD2	cacaatcac---	AAATC	A	GAG	CTT	TAAT	CAG	GCA	AAT	CAC	CT	CA	AGC	GT	T	
h-MAD2	ACA	TTT	CTG	CC	actgt	tgga	agttt	ctt	gt	tca	t	ttg	atc	t	gctga	t
y-MAD2	ACC	TTT	CTG	CC	cgaac	taac	aaa	gaag	gt	ggg	t	acac	at	t	cacag	t
h-MAD2	tacagacaaaga	ttt	gg	t	gtg	tac	ct	gaaaa	tg	g	gaa	ga	gt	ggg	gac	cac
y-MAD2	tgcataatacaya	cgc	gg	atg	ctaaa	gtt	ccg	tta	gaa	tg	ggg	ccg	act	cca		
h-MAD2	agtt	ta	ttacc	aatt	ctga	ggaa	gtgc	gcc	ttc	gt	t	cattt	ac	tacta	ca	
y-MAD2	atag	ta	aagag	atac	ctga	tgg	gtg	aagtag	ttc	aa	t	tcaaa	ac	attct	ct	
h-MAD2	atccacaaagta	aatag	catg	gtg	gccta	caaaa	t	cctgt	ca	atg	actg					
y-MAD2	accacgatcat	aaag	t	ggt	gcg	caggt	ca	gcta	taaat	att	aa	-----				

h-MAD2 a
y-MAD2 -

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FIG. 3A

TGGAAGCGCGTGCTTTTGTGTTGTGCCCTGGCCATGGCGCTGCAGCTCTCCCGGGAGCAG
M A L Q L S R E Q 9

GGAATCACCCCTGCGCGGGAGCGCCGAAATCGTGGCCGAGTTCTTCTCATTTCGGCATCAAC
G I T L R G S A E I V A E F F S F G I N 29

AGCATTTTATATCAGCGTGGCATATATCCATCTGAAACCTTTACTCGAGTGCAGAAATAC
S I L Y Q R G I Y P S E T F T R V Q K Y 49

GGACTCACCTTGCTTGTAACACTGATCTTGAGCTCATAAAATACCTAAATAATGTGGTG
G L T L L V T T D L E L I K Y L N N V V 69

GAACAACTGAAAGATTGGTTATACAAGTGTTTCAGTTCAGAACTGGTTGTAGTTATCTCA
E Q L K D W L Y K C S V Q K L V V V I S 89

AATATTGAAAGTGGTGAGGTCTGGAAAGATGGCAGTTTGATATTGAGTGTGACAAGACT
N I E S G E V L E R W Q F D I E C D K T 109

GCAAAAGATGACAGTGCACCCAGAGAAAAGTCTCAGAAAGCTATCCAGGATGAAATCCGT
A K D D S A P R E X S Q K A I Q D E I R 129

TCAGTGATCAGACAGATCACAGCTACGGTGACATTTCTGCCACTGTTGGAAGTTTCTTGT
S V I R Q I T A T V T F L P L L E V S C 149

TCATTTGATCTGCTGATTTATACAGACAAAGATTTGGTTGTACCTGAAAAATGGGAAGAG
S F D L L I Y T D K D L V V P E K W E E 169

TCGGGACCACAGTTTATTACCAATTCTGAGGAAGTGCGCCTTCGTTTACTACTACA
S G P Q F I T N S E E V R L R S F T T T 189

ATCCACAAAGTAAATAGCATGGTGGCCTACAAAATTCCTGTCAATGACTGAGGATGACAT
I H K V N S M V A Y K I P V N D * 205

GAGGAAAATAATGTAATTGTAATTTTGAAATGTGGTTTTCTGAAATCAGGTCATCTATA
GTTGATATGTTTTATTTTCATTGGTTAATTTTACATGGAGAAAACCAAATGATACTTAC
TGAAGTGTGTGTAATTGTTCCCTTTTATTTTTTTGGTACCTATTTGACTTACCATGGAGTT
AACATCATGAATTTATTGCACATTGTTCAAAAGGAACCAGGAGGTTTTTTTGTCACATT
GTGATGTATATTCCTTTGAAGATAGTAACTGTAGATGGAAAACTTGTGCTATAAAGCTA
GATGCTTTTCTAAATCAGATGTTTTGGTCAAGTAGTTTGACTCAGTATAGGTAGGGAGAT
ATTTAAGTATAAAATACAACAAAGGAAGTCTAAATATTCAGAATCTTTGTAAAGTCTCTG
AAAGTAACTCATAATCTATAAACAATGAAATATTGCTGTATAGCTCCTTTTGACCTTCAT
TTCATGTATAGTTTTCCCTATTGAATCAGTTTCCAATTATTTGACTTTAATTTATGTAAC
TTGAACCTATGAAGCAATGGATATTTGTACTGTTTAAATGTTCTGTGATACAGAACAGATT
AATACTCCCTTTTTATCATTACAGTTAGCTAAAAAATTGCCAGGCAGTCCACAAAACAGA
ATTTGCTTTAAGACCAACCCACAGAGTCAGCTGGAGACTAACGGCGCTGGGGCCTGCTGG
GCCGGGATATAGTCGTGTTTAGCTAAGTGTGAGAGCATTAAGAAGAAAGTCCTGGTTGG
AGGCGCAAGGCCTGCAGCACCAGCTGTGGAATCCCCAATAATGT

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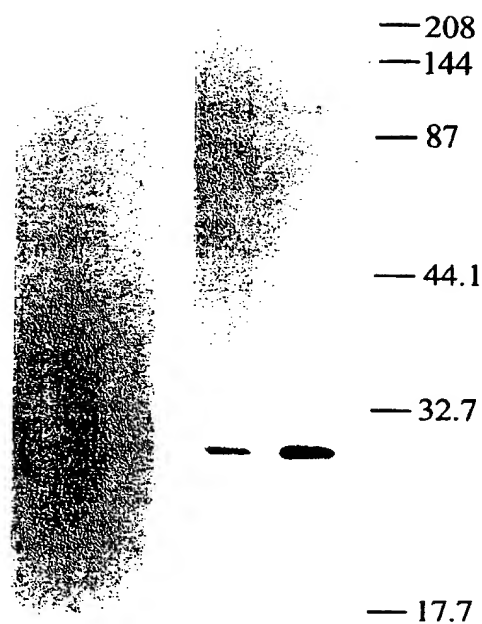
FIG. 3B

hsMAD2	1	M	A	L	Q	L	S	R	E	Q	G	I	T	L	R	G	S	A	E	I	V	A	E	F	F	S	F	G	I	N	S	I	L	Y	33	
XMAD2	1	M	A	G	Q	L	T	R	-	E	G	I	T	L	K	G	S	A	E	I	V	S	E	F	F	F	C	G	I	N	S	I	L	Y	32	
scMAD2	1	-	-	-	-	-	-	-	M	S	Q	S	I	S	L	K	G	S	T	R	T	V	T	E	F	F	E	Y	S	I	N	S	I	L	Y	27
hsMAD2	34	Q	R	G	I	Y	P	S	E	T	F	T	R	V	Q	K	Y	G	L	T	L	L	V	T	T	D	L	E	L	I	K	Y	L	N	66	
XMAD2	33	Q	R	G	I	Y	P	S	E	T	F	T	I	R	Q	K	Y	G	L	T	L	L	V	S	T	D	P	A	L	K	E	Y	L	N	65	
scMAD2	28	Q	R	G	V	Y	P	A	E	D	F	V	T	V	K	K	Y	D	L	T	L	L	K	T	H	D	D	E	L	K	D	Y	I	R	60	
hsMAD2	67	N	V	V	E	Q	L	K	D	W	L	Y	K	C	S	V	Q	K	L	V	V	I	S	N	I	E	S	G	E	V	L	E	R	99		
XMAD2	66	K	V	T	D	Q	L	K	D	W	L	Y	K	C	Q	V	Q	K	L	V	V	I	T	S	I	D	S	N	E	I	L	E	R	98		
scMAD2	61	K	I	L	L	Q	V	H	R	W	L	L	G	G	K	C	N	Q	L	V	L	C	I	V	D	K	D	E	G	E	V	E	R	93		
hsMAD2	100	W	Q	F	D	I	E	C	D	K	T	A	K	D	S	A	P	R	E	K	S	Q	K	A	I	Q	D	E	I	R	S	V	I	132		
XMAD2	99	W	Q	F	D	I	E	C	D	K	T	V	K	D	G	-	I	V	R	E	K	S	Q	K	V	I	Q	E	E	I	R	S	V	I	130	
scMAD2	94	W	S	F	N	V	Q	H	I	S	G	N	S	N	G	-	Q	D	D	V	V	D	L	N	T	T	Q	S	Q	I	R	A	L	I	125	
hsMAD2	133	R	Q	I	T	A	T	V	T	F	L	P	L	L	E	V	S	-	-	C	S	F	D	L	L	I	Y	T	D	K	D	L	V	163		
XMAD2	131	R	Q	I	T	A	T	V	T	F	L	P	L	L	E	T	A	-	-	C	A	F	D	L	L	I	Y	T	D	K	D	L	E	V	161	
scMAD2	126	R	Q	I	T	S	S	V	T	F	L	P	E	L	T	K	E	G	Y	T	F	T	V	L	A	Y	T	D	A	D	A	K	V	158		
hsMAD2	164	P	E	K	W	E	E	S	G	P	Q	F	I	T	N	S	E	E	V	R	L	R	S	F	T	T	I	H	K	V	N	S	M	196		
XMAD2	162	P	E	K	W	E	E	S	G	P	Q	F	V	S	N	S	E	E	V	R	L	R	S	F	T	T	I	H	K	V	N	S	M	194		
scMAD2	159	P	L	E	W	A	D	S	N	S	K	E	I	P	D	G	E	V	V	Q	F	K	T	F	S	T	N	D	H	K	V	G	A	Q	191	
hsMAD2	197	V	A	Y	K	I	P	V	N	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	205		
XMAD2	195	V	A	Y	K	K	I	D	T	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	203		
scMAD2	192	V	S	Y	K	Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	196		

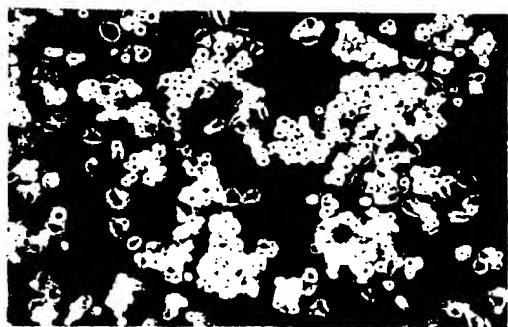
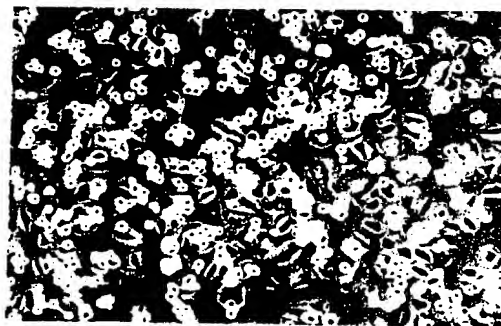
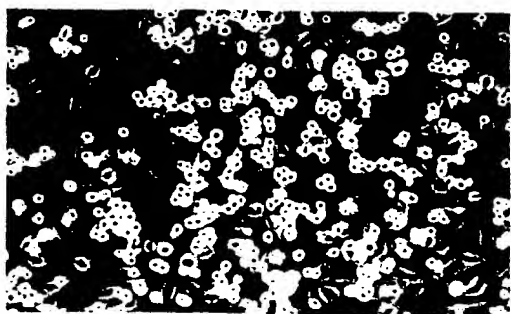
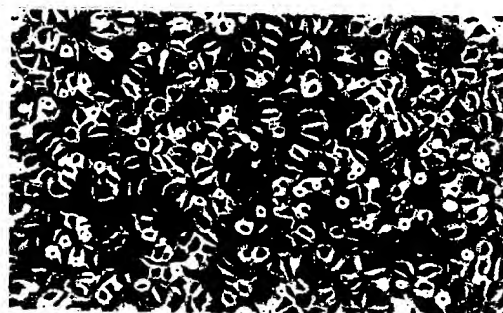
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FIG. 3C

Preimmune α -hsMad2 Δ α -hsMad2
1 2 3 4 5 6



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FIG. 4A-1
Buffer α -hsMad2 Δ **FIG. 4A-2**
Preimmune α -hsMad2**FIG. 4A-3****FIG. 4A-4**

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FIG. 5A-1
Preimmune

FIG. 5A-2
 α -hsMAD2 Δ

FIG. 5A-3
 α -hsMAD2

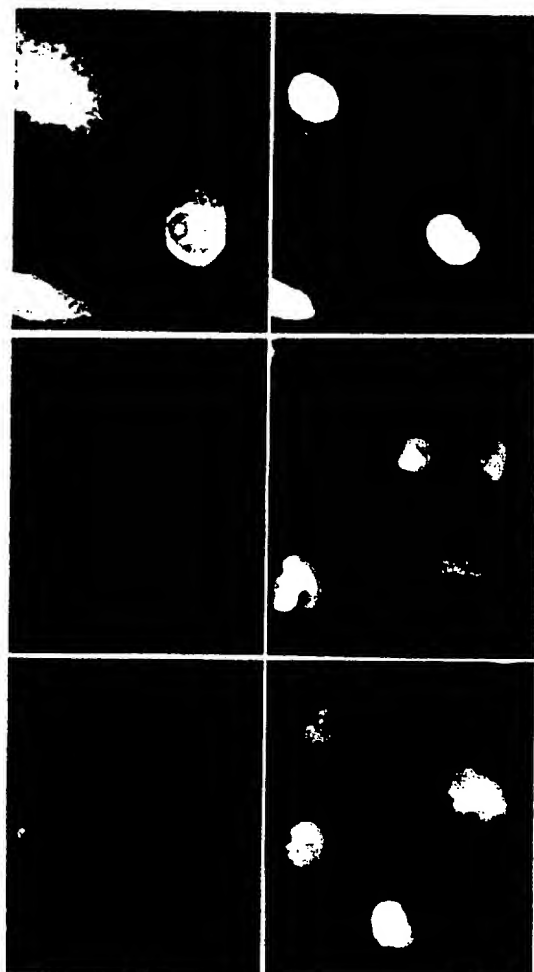
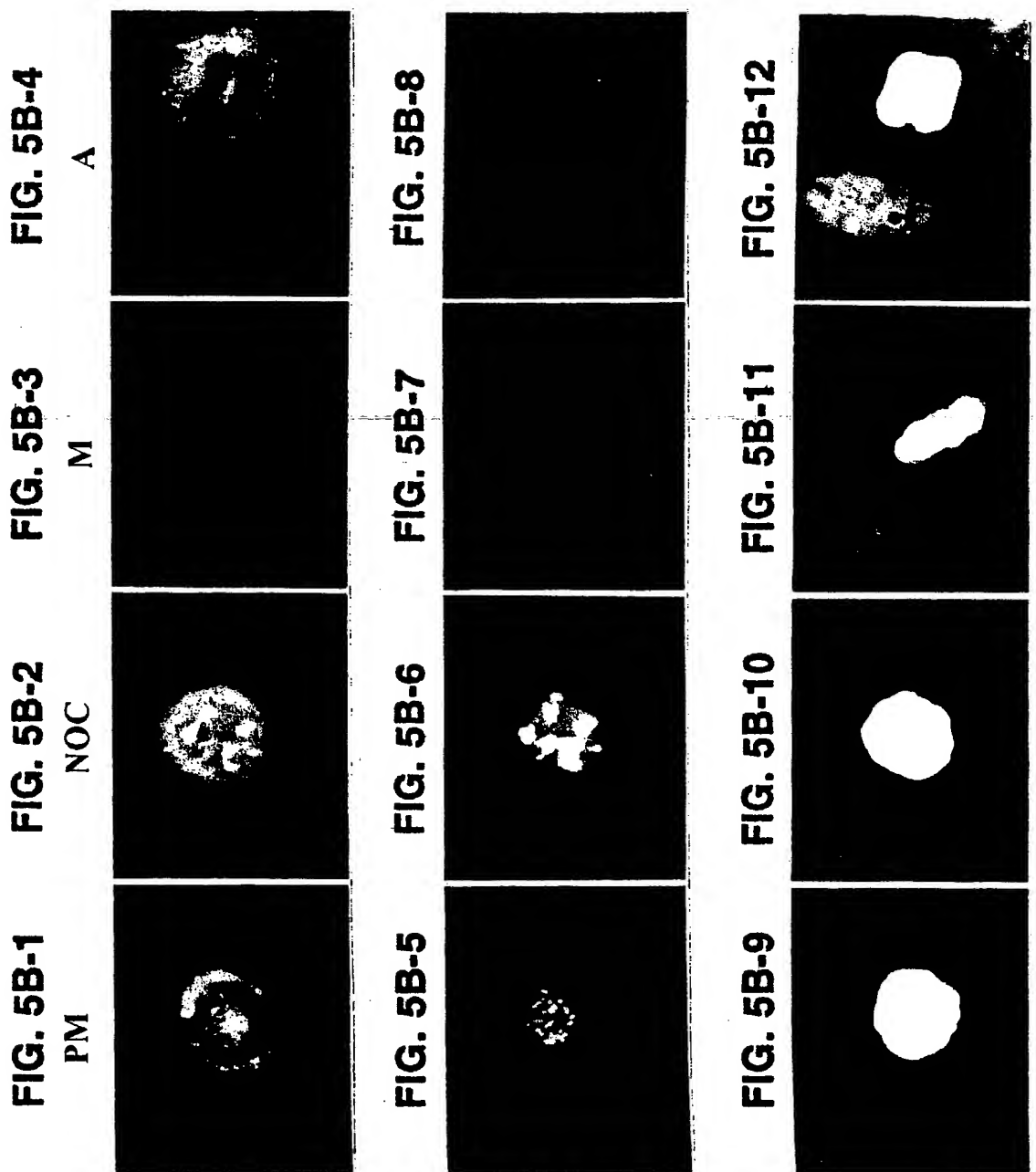


FIG. 5A-4

FIG. 5A-5

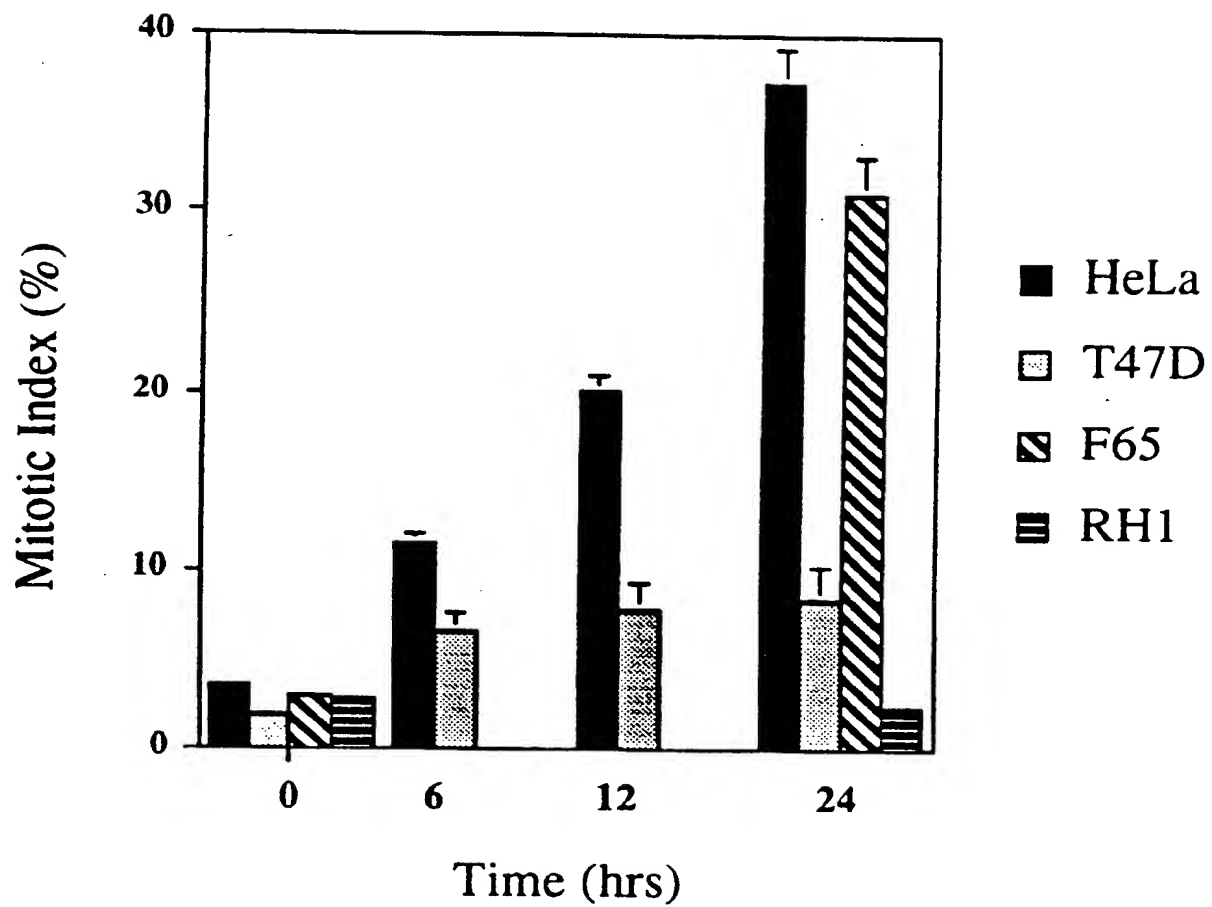
FIG. 5A-6

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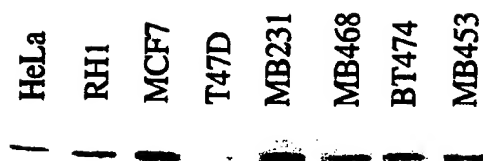
FIG. 6A



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FIG. 6B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12021

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : 536/23.1, 24.5; 435/6, 240.2, 320.1; 530/350; 424/130.1; 436/500; 514/44; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.5; 435/6, 240.2, 320.1; 530/350; 424/130.1; 436/500; 514/44; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ARMSTRONG et al. cDNA Cloning and Expression of the α and β Subunits of Rat Rab Geranylgeranyl Transferase. Journal of Biological Chemistry. 05 June 1993, Vol. 268, No. 16, pages 12221-12229, see entire document.	1-55
Y	LI et al. The mitotic feedback control gene <i>MAD2</i> encodes the α -subunit of a prenyltransferase. Nature. 04 November 1993, Vol. 366, pages 82-84, see entire document.	1-55
Y	BROWN et al. Mad Bet for Rab. Nature. 04 November 1993, Vol. 366, pages 14-15, see entire document.	1-55

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 SEPTEMBER 1996

Date of mailing of the international search report

02 OCT 1996

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/12021

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JIANG et al. Bet2p and Mad2p are components of a prenyltransferase that adds geranylgeranyl onto Ypt1p and Sec4p. Nature. 04 November 1993, Vol. 366, pages 84-86, see entire document.	1-55

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/12021

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07H 21/04; C12N 15/00, 15/63, 15/85, 15/86; C07K 14/00; A61K 39/395, 48/00; G01N 33/53; C12Q 1/68

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